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THE EFFECT OF DENATURATION OF PROTEINS ON THEIR COACERVATION WITH DYES

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Frog serum and horse serum as well as serum albumen and egg albumen are used to study the effect of denaturation on the coacervation capacity of proteins so as to determine if cessation of granule formation results from denaturation charges in damaged cells which prevent coacervation. Among factors inducing denaturation the author studied acetone, temperature, ultraviolet rays and urea. It was found that urea in no way effects coacervation.

Denaturation changes prohibit protein from entering into a coacervation reaction with dyes or other substances. Therefore, granule formation in an injured cell may cease due to denaturation of protoplasm proteins resulting from disruption in the metabolism.

During the lifetime of an organism certain basic pigments are deposited as /1139* granules in the protoplasm of undamaged cells. When the cells are damaged and even when this damage is reversible, the capacity of a cell to form granules is destroyed. D. N. Nasonov and V. Ya. Aleksandrov (refs. 1 and 2) have shown that as a result of this, the coloration by pigments which form granules during the lifetime of an organism may be used to judge the functional state of cells and tissues. It is therefore of interest to investigate the mechanism associated with the granular accumulation of pigments in the cell. This is particularly so because substances normally encountered in an organism are also deposited in a similar manner.

In the past it has been shown that pigment granules form as a result of the interaction of the pigment with some protoplasmic protein (refs. 3-5). It has been possible for us to establish that the basis of this process lies in the complex coacervation of pigments with protein (ref. 6). However, in this connection we are faced with the following problem: if the granules occur due to the colloidal-chemical reaction of coacervation, how do we explain the fact that they are formed only in a cell which is functioning normally and that after a cell is damaged the formation of granules becomes impossible? According to the denaturation theory of Nasonov and Aleksandrov, the action of various alternating agents on the cell is responsible for a series of alterations in the protoplasm which are produced by the denaturation of cell proteins. The thought occurs as to whether the native state of protoplasmic protein, which is maintained by the vital activity of the cell, is a necessary condition for coacervation. If this is so, the cessation of granule formation when the cell is damaged may be looked upon as a result of denaturation changes which make the

*Numbers given in margin indicate pagination in original foreign text.

[1]Presented by academician N. N. Anichkov, 1 September 1950.

coacervation reaction impossible. In this case the cessation of granule formation may also serve as an indicator of cell protein denaturation, which to date has been the increase in the sorption properties (refs. 1 and 7). In order to study this problem we started to investigate the effect of denaturation on the coacervation capacity of proteins.

Investigations were carried out using frog and horse serum, serum albumen and egg albumen. In the last two cases experiments were carried out in a phosphate buffer and in the MacIlvain buffer in the pH interval from 6.5 to 8. The protein concentration was 0.1 percent. Among the dyes used, the principal one was Nile blue sulfate (0.1 percent) and sometimes neutral red (0.1 percent). Coacervation was achieved by carefully combining droplets of protein and dye on a glass slide with a glass rod (ref. 6). Of the factors which induced the denaturation action we investigated the following: acetone, temperature, ultraviolet rays and urea.

Acetone, first of all, produces colloidal changes in the protein. At low /1140
temperatures the coagulation produced by acetone is easily reversible (ref. 1). At room temperature coagulation becomes irreversible because there are additional effects produced by molecular changes in the protein. We utilized these properties of acetone to establish the effect of variation in the colloidal state of the protein on its capacity to coacervate.

The experiments were conducted in the following manner: 0.3 ml of acetone was added to 1 ml of 1 percent egg albumen at room temperature. In this manner the acetone concentration was equal to 23 percent. Under the influence of acetone the protein precipitated and during reaction with the dye (coacervation) did not take place. After 30 minutes, 3.7 ml of water was added to this mixture and consequently the concentration of acetone dropped to 6 percent. In spite of this the protein residue did not dissolve and the capacity to coacervate was not reestablished. It was possible to assume that the presence of acetone alone, even at a concentration of 6 percent, inhibits coacervation. We therefore conducted a series of control experiments at 0°C. At 0°, just as at room temperature, protein was precipitated in the presence of 23 percent acetone and was incapable of producing coacervation. However, if in this case, after 30 minutes the acetone concentration was reduced to 6 percent by the addition of water, the residue dissolved almost completely and the resulting solution of native protein reestablished its capacity to produce coacervation. Consequently acetone in itself does not inhibit coacervation. However, the colloidal changes produced in the protein by acetone make coacervation impossible.

Temperature. The protein solution was heated in a water bath for 10 minutes at various temperatures. After it was cooled its coacervation capacity was investigated. Unheated protein was used for the control. In the control sample the mixing of protein and dye drops immediately produces coacervation. In the experiment, i.e., after the heating of the protein, drops of coacervate are not formed. Instead of this, when protein is mixed with a dye, the large and small flakes of coagulated protein become colored. The results of these experiments are shown in table 1. The frog serum loses its capacity to coacervate after it is heated for ten minutes at 55°. Horse serum ceases to coacervate after it is heated to 65°. However, the nature of coacervation changes

TABLE 1. THE EFFECT OF THERMAL, ULTRAVIOLET AND RADIANT DENATURATION OF PROTEINS ON THEIR COACERVATION WITH NILE BLUE SULFATE.

	pH	Temperature, °C							Ultraviolet rays				Urea in moles			
									0°		20°					
		50	55	60	65	70	75	80	1 hr	2 hr	1 hr	2 hr	4	6	8	10
Blood serum from a frog		+	-	-									-	-	-	-
Blood serum from a horse			+	+	-	-							+	+	-	-
Serum albumen	6.5			+	+	-	-	-					+	-	-	-
	7.0			+	+	-	-	-					+	-	-	-
	7.4			+	+	+	-	-	+	±	+	±	+	-	-	-
	8.0			+	+	+	+	-		-		-	+	-	-	-
Egg albumen	6.5				+	-	-						±	-	-	-
	7.0				+	-	-			±		±	±	-	-	-
	7.4				+	-	-			-	+	-	±	-	-	-
	8.0				+	-	-		+				±	-	-	-

transparent: Serum albumen, pH 7.0, 60°C; pH 7.4, 60°C; pH 8.0, 60°C
transparent: Serum albumen, pH 7.0, 65°C; pH 7.4, 65°C; pH 8.0, 65°C
transparent: Serum albumen, pH 7.0, 70°C; pH 7.4, 70°C; pH 8.0, 70°C
turbid: Serum albumen, pH 6.5, 75°C; pH 7.0, 75°C
transparent: Serum albumen, pH 7.4, 75°C; pH 8.0, 75°C
turbid: Serum albumen, pH 6.5, 80°C; pH 7.0, 80°C
transparent: Serum albumen, pH 7.4, 80°C; pH 8.0, 80°C
turbid: Egg albumen, pH 7.0, 65°C; pH 7.4, 65°C
turbid: Egg albumen, pH 7.0, 70°C; pH 7.4, 70°C
turbid: Egg albumen, pH 7.0, 75°C; pH 7.4, 75°C

Note. + sign indicates a large number of coacervate droplets, ± sign indicates individual drops of coacervate, - sign indicates that coacervation is absent.

only after exposure to a temperature of 60°. In addition to normal droplets of coacervate, a large number of flakes are encountered, as well as net-like formations containing a large number of drop-like swellings. Apparently these are formed by the union and extension of individual droplets of the coacervate.

The coagulation of the protein, which has been denatured by heat, may be looked upon as the results of its molecular changes. From the very determination of coacervation it follows that coagulated protein is incapable of coacervation. Therefore the results which we have obtained in experiments with the action of acetone on egg albumen and on heating of serums are not unexpected. As we know, under certain conditions it is possible to obtain denaturation changes of molecules without subsequent changes in the protein's colloidal state. In this connection the question arises as to whether protein which has been denatured but not coagulated is capable of coacervation. We carried out a series of experiments under conditions which excluded colloidal transformations in the protein. Specifically this involved heating at pH values sufficiently

remote from the isoelectric point. In these experiments we used serum albumen and egg albumen.

At pH 6.5 and 7.0, serum albumen, after heating to 70°, ceased to coacervate, although the solutions in this case remained transparent. At pH 7.5 and 8.0 the protein turned out to be more stable to temperature. However, as we can see from table 1, the capacity to coacervate always disappeared before turbidity appeared. The same experiments carried out with egg albumen produced/1141 no results. Egg albumen became turbid at all values of pH to the same degree and since it was already turbid, i.e., partially coagulated, it retained its capacity to coacervate when heated below 70°. Above 70° the egg albumen lost its capacity to coacervate.

Thus, in experiments with frog serum and horse serum and with egg albumen denaturation, which accompanied changes of the colloidal state, made the protein incapable of coacervation. On the other hand, in experiments with serum albumen molecular alterations were entirely sufficient for this purpose.

At room temperature ultra-violet rays produce molecular as well as colloidal changes in protein. However, if irradiation is carried out at 0° the molecular alterations are not accompanied by colloidal alterations (ref. 9). Therefore experiments on the action of radiant energy were conducted both at room temperature and at 0°. A drop of serum albumen or egg albumen with a concentration of 0.1 percent in a phosphate buffer (pH 7.4) was placed on the slide with a depression, covered with a quartz coverslip and illuminated with a mercury quartz lamp. Illumination for one hour did not inhibit coacervation. After a two-hour period of illumination with ultraviolet rays, coacervation ceased almost completely (table 1). However, in a series of experiments we still observed the occurrence of very small individual droplets of coacervate. We can see from table 1 that the results of the experiments at room temperature and at 0° are entirely identical. Consequently, protein which has been denatured by ultra-violet rays loses its capacity to coacervate with pigments even in the case when the changes which take place in it are limited to molecular alterations and do not affect the colloidal state as in the experiments at 0°.

The action of urea differs from the action of temperature and ultraviolet rays in that, while denaturing the protein, it inhibits its coagulation. Therefore, the investigation of the effect of urea on the coacervation capacity of protein is of particular interest.

Urea was added to a 0.1 percent solution of protein in a phosphate buffer, in solid form and in a quantity such that its final concentration was 4, 6, 8 and 10 molar. After 15 minutes and after one hour the coacervation capacity of such solutions was investigated. Since urea apparently acts very quickly on the protein, the results of observations conducted after 15 minutes and after one hour did not differ (table 1). Frog serum and egg albumen lose their capacity to coacervate in 4 M urea solutions. In a 6 M solution the serum albumen ceases to coacervate. Finally, in an 8 M solution of urea the coacervation of horse serum ceases. Instead of the large number of intensely dyed droplets of coacervate, which result from reaction of dye and protein in the absence of urea,

in all these cases the liquids mix uniformly. Only occasionally can we notice very small, weakly colored flakes. It was possible to surmise that the presence of urea in the solution inhibits the coacervation reaction independently of its action on the protein. To verify this proposition we investigated the coacervation of nonprotein colloidal solutions (gum arabic and sodium nucleate) in the presence of the same urea concentrations. It turned out that even a 10 M solution of urea does not inhibit the coacervation of gum arabic and sodium nucleate. This shows that the presence of urea in itself has no effect on coacervation.

Thus in the case of action by urea and also in the case when heating takes place in an alkali medium and irradiation with ultraviolet rays is carried out at low temperatures, the denaturation changes of protein molecules, which are not even complicated by coagulation, deprive the protein of its capacity to /1142 enter into a coacervation reaction with dyes. Denatured protein is apparently incapable of coacervation not only with dyes but with other substances as well. This is supported by the observations of Bungenberg de Jong (refs. 10 and 11), who showed that a complex coacervate of serum albumen with gum arabic and with clupein gradually breaks down due to the denaturation of the protein component.

Thus the proteins which are denatured by acetone, temperature, ultraviolet rays and urea lose their capacity to coacervate. This gives us a basis to assume that granule formation in a cell during injury may cease due to the denaturation of protoplasm proteins which is produced directly by the action of the agent or which occurs as a result of disruption in metabolism necessary to maintain the cell proteins in their native state.

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